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Berberine Blocks the Relapse of *Clostridium difficile* Infection in C57BL/6 Mice after Standard Vancomycin Treatment

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Vancomycin is a preferred antibiotic for treating *Clostridium difficile* infection (CDI) and has been associated with a rate of recurrence of CDI of as high as 20% in treated patients. Recent studies have suggested that berberine, an alternative medical therapy for gastroenteritis and diarrhea, exhibits several beneficial effects, including induction of anti-inflammatory responses and restoration of the intestinal barrier function. This study investigated the therapeutic effects of berberine on preventing CDI relapse and restoring the gut microbiota in a mouse model. Berberine was administered through gavage to C57BL/6 mice with established CDI-induced intestinal injury and colitis. The disease activity index (DAI), mean relative weight, histopathology scores, and levels of toxins A and B in fecal samples were measured. An Illumina sequencing-based analysis of 16S rRNA genes was used to determine the overall structural change in the microbiota in the mouse ileocecum. Berberine administration significantly promoted the restoration of the intestinal microbiota by inhibiting the expansion of members of the family *Enterobacteriaceae* and counteracting the side effects of vancomycin treatment. Therapy consisting of vancomycin and berberine combined prevented weight loss, improved the DAI and the histopathology scores, and effectively decreased the mortality rate. Berberine prevented CDIs from relapsing and significantly improved survival in the mouse model of CDI. Our data indicate that a combination of berberine and vancomycin is more effective than vancomycin alone for treating CDI. One of the possible mechanisms by which berberine prevents a CDI relapse is through modulation of the gut microbiota. Although this conclusion was generated in the case of the mouse model, use of the combination of vancomycin and berberine and represent a novel therapeutic approach targeting CDI.

Clostridium difficile infection (CDI) arises in the setting of antibiotic administration, where the disruption of the normal indigenous gut microbiota leads to susceptibility to *C. difficile* colonization and colitis. Vancomycin and metronidazole have been used to treat CDI for the past 25 years, but approximately 20% of treated patients develop recurrent disease (1). Given the poor efficacy of vancomycin treatment for frequent recurrences of CDIs, researchers in the field have actively sought treatment alternatives for several decades (2). To minimize CDI recurrences, new efforts are directed at not only killing *C. difficile* but also combining antimicrobials with two other major approaches: preservation of the intestinal microbiota and optimization of the immune response to CDI and toxins (3).

Berberine, an isoquinoline alkaloid, has widely been used as an herbal medicine to treat gastrointestinal (GI) disorders, such as gastroenteritis, bacterium-associated diarrhea, and intestinal parasitic infections, for thousands of years in China (4). The pharmacological actions of berberine include metabolic inhibition of certain organisms, bacterial enterotoxin formation, intestinal fluid accumulation, ion secretion, and smooth muscle contraction and reduction of the inflammation and agitation that contribute to bile and bilirubin secretion (4, 5). Recent studies have suggested that berberine promotes recovery from colitis and inhibits inflammatory responses to colonic macrophages and epithelial cells in dextran sulfate sodium-treated mice (6). However, the effect of berberine treatment on CDI has not been explored or discussed in the literature.

Previous studies have shown that vancomycin treatment results in collateral damage to the gut microbiota, including a decrease in the populations of the phyla *Firmicutes* and *Bacteroidetes*, which was accompanied by a corresponding increase in the population of *Proteobacteria* (7). One possible approach to minimizing such collateral damage would be utilizing some modality that shows a specific inhibition of the expansion of the population of *Proteobacteria*. Berberine has been suggested to inhibit the overgrowth of organisms, such as members of the family *Enterobacteriaceae*, while it has no effect on indigenous lactobacilli and bifidobacteria (8, 9). The unique properties of berberine make it a potential therapeutic candidate that may target certain bacterial populations, restore the microbiota in the colon, and mitigate the collateral damage caused by vancomycin. Our study aims to investigate the potential therapeutic efficacy of the combination of berberine and vancomycin in restoring the gut microbiota in a mouse model of CDI.

MATERIALS AND METHODS

Reagents. Berberine chloride (purity by high-pressure liquid chromatography, ≧98.0%) was obtained from J&K Scientific, Ltd. Antibiotics were

Received 23 November 2014 Returned for modification 15 January 2015
Accepted 22 March 2015

Accepted manuscript posted online 30 March 2015

Citation Lv Z, Peng G, Liu W, Xu H, Su J. 2015. Berberine blocks the relapse of *Clostridium difficile* infection in C57BL/6 mice after standard vancomycin treatment. Antimicrob Agents Chemother 59:3726–3735. doi:10.1128/AAC.04794-14.

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doi:10.1128/AAC.04794-14

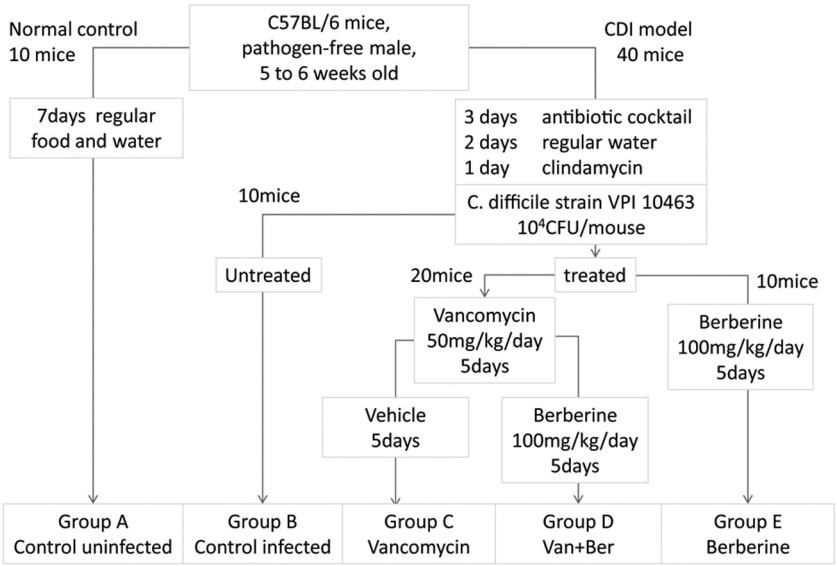

FIG 1 Experimental design for study of mice with CDI receiving different treatments. Mice in group A received regular food and water and were not infected with *C. difficile*, mice in group B were pretreated with the antibiotic mixture 3 days before clindamycin administration and challenge with *C. difficile* at 10^4 CFU, mice in group C were infected and treated with vancomycin for 5 days, mice in group D were infected and treated with the combination of berberine (Ber) and vancomycin (Van), and mice in group E were infected and treated with berberine for 5 days.

obtained from Sigma-Aldrich. *C. difficile* strain VPI 10463 (ATCC 43255) was purchased from ATCC. *C. difficile* agar base, selective supplements, and defibrinated horse blood were purchased from Oxoid Ltd. (Oxoid, Thermo Fisher Scientific Inc., Basingstoke, United Kingdom). A QIAamp DNA stool minikit was purchased from Qiagen (Qiagen China [Shanghai] Co., Ltd.).

Animals. Pathogen-free male C57BL/6 mice (age, 5 to 6 weeks) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd., (Beijing, China). Five or fewer mice were housed per cage under conventional conditions at the Animal Center of the Beijing Friendship Hospital, Capital Medical University (Beijing, China), for 1 week before initiating the study. All experiments were performed according to protocols approved by the Animal Studies Subcommittee of the Capital Medical University.

Mouse model of CDI. For the infection model, we used a modified version of the published protocol of Chen et al. (10). An antibiotic mixture of kanamycin (0.4 mg/ml), gentamicin (0.035 mg/ml), colistin (850 U/ml), metronidazole (0.215 mg/ml), and vancomycin (0.045 mg/ml) was prepared in the drinking water. Ten mice in each group were fed the water containing the antibiotic mixture for 3 days. Then, all mice were given regular autoclaved water for 2 days and received a single dose of clindamycin (10 mg/kg of body weight) intraperitoneally for 1 day before *C. difficile* challenge. The stock suspension of *C. difficile* strain VPI 10463 (ATCC 43255), which produces both toxins A (Tcd A) and B (Tcd B), was anaerobically cultured with *C. difficile* agar base (Oxoid) and grown for 24 to 48 h at 37°C (11), before being administered intragastrically. The day on which the mice were orally administered *C. difficile* (10^4 CFU/mouse) was set as day 0. During the experiment, the mice were monitored daily for signs of disease, such as diarrhea, hunched posture, and fecal toxins A and B, and their mean relative weight was also monitored.

Experimental scheme. To establish a mouse model of CDI and evaluate treatment, 50 mice were randomly divided into 5 groups: group A, consisting of control uninfected mice receiving regular food and water without any treatment; group B, consisting of control infected mice receiving pretreatment with a 3-day course of the antibiotic mixture and *C. difficile* challenge at day 0; group C, consisting of infected mice treated with vancomycin for 5 days; group D, consisting of infected mice treated with vancomycin for 5 days, followed by berberine for 5 consecutive days; and group E, consisting of infected mice treated with berberine alone for 5 days. In the primary infection, mice were challenged with *C. difficile* at 10^4 CFU/mouse after administration of the antibiotic mixture and clindamycin. Vancomycin treatment (50 mg/kg/day via gavage) was started on day 1 after *C. difficile* challenge in group C and D mice and continued for 5 days. To investigate the potential synergy between vancomycin and berberine, after 5 days of vancomycin treatment, we initiated berberine treatment in a portion of the vancomycin-treated mice. Berberine (100 mg/kg/day) was administered by gavage to group D mice after 5 days of vancomycin treatment. As a comparison, the same dosage of berberine was administered by gavage to group E mice after *C. difficile* challenge. The experimental scheme is illustrated in Fig. 1. Animals were monitored for death (see Fig. 2), weight loss (see Fig. 3), and the optical density (OD) of toxins A and B (Fig. 4) from day 0 to day 34.

Mean relative weight and mouse DAI. Mean relative weight and the disease activity index (DAI) were assessed daily. The surviving mice were weighed every day, and relative body weights are presented as the percentage of the original mean body weight on day 0. The DAI was a modification of a scoring system previously described for colitis (12). The index uses scales from 0 to 4 classifying the loss of body weight (0 = none, 1 = 1% to 5%, 2 = 5% to 10%, 3 = 10% to 20%, and 4 >20%), the consistency of the feces (0 = normal feces, 2 = loose feces, 4 = diarrhea), and blood in feces (0 = normal, 2 = fecal blood positive, 4 = gross blood). The DAI is the sum of the three scores divided by three.

Histopathological analysis. To assess the histopathology score and bacterial community, 60 mice were divided into 4 groups (the control infected group, vancomycin group, vancomycin-berberine-treated group, berberine-treated group). Animals that were in a moribund state were euthanized. In addition, animals were euthanized at the end of each experimental stage: stage 0, after 3 days of antibiotic treatment and before *C. difficile* challenge; stage 1, at day 5 after vancomycin or berberine treatment; stage 2, at day 11 after treatment with vancomycin combined with berberine; stage 3, at day 21; stage 4, at day 28; and stage 5, at day 34 (the end of the experimental period). Colon tissue samples were collected for histopathological analysis (see Fig. 5 and 6).

The colon tissue samples were collected for histopathological analysis and evaluated for mucosal damage and inflammation induced by toxins A and B. The resected colon or cecum tissues were fixed in 4% formaldehyde

buffered with phosphate-buffered saline and then embedded in paraffin. The 6-μm-thick sections were stained with hematoxylin and eosin (H&E) for histological analysis. Tissues were reviewed in a blinded fashion and assessed by use of a previously validated gastrointestinal histologic inflammatory score (10). All sections were read and scored independently by three pathologists, and the average scores were used as the final scores. For mice with CDIs, histology scores consisted of 13 levels, from 0 to 12. The total score was calculated as the sum of the scores for the following 4 parameters: (i) neutrophil migration and tissue infiltration, (ii) hemorrhagic congestion, (iii) edema of the mucosa, and (iv) epithelial cell damage. Each parameter was graded and given a score of from 0 to 3, where 0 indicates none, 1 indicates mild, 2 indicates moderate, and 3 indicates severe (8).

***C. difficile* toxin assay.** To determine the level of viable vegetative and spore forms of *C. difficile* and how these were impacted by the different therapies studied, *C. difficile* toxins A and B were detected in 200 mg of semisolid or solid fecal samples using a *C. difficile* toxin A and B kit (bioMérieux, Inc., France) and a Vidas immunoassay system. The assay is an automated test for the qualitative detection of *C. difficile* toxins A and B in fecal specimens using the enzyme-linked fluorescence assay (ELFA) technique. The test value for each sample is calculated by the Vidas instrument as follows: test value = patient relative fluorescence value (RFV)/standard RFV. Interpretation of the test value is as follows: <0.13, negative result; ≥0.13 to <0.37, equivocal result; ≥0.37, positive result.

DNA extraction, PCR amplification, and Illumina sequencing. We next assessed the shifts in the microbial community structure and composition associated with CDI and the different treatment schemes. At the end of the treatment period (1 day after *C. difficile* challenge, after 5 days of vancomycin or berberine administration, after 5 days of vancomycin administration followed by 5 days of berberine administration), four mice in each group were euthanized. The ileocecal contents of the euthanized mice were collected for DNA extraction, PCR amplification, amplicon quantification, pooling, and sequencing (13). Bacterial genomic DNA was extracted from samples using the QIAamp DNA stool minikit (Qiagen China [Shanghai] Co., Ltd.), according to the manufacturer's instructions. Consensus primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACCAGGGTATCTAAT-3′) were used to amplify the V3 and V4 regions of bacterial 16S rRNA. A bar code and adapter were incorporated between the adapter and the forward primers. The PCR amplification was carried out in triplicate using a 20-μl reaction mixture with 0.6 mM each primer, 10 to 50 ng of template DNA, 4 μl of the PCR buffer, and 2.5 U of Phusion DNA polymerase. The amplification program consisted of an initial denaturation step at 95°C for 2 min, followed by 25 cycles, where 1 cycle consisted of 95°C for 30 s (denaturation), 55°C for 30 s (annealing), and 72°C for 45 s (extension). A final extension of 72°C was performed for 10 min, and the mixture was held at 10°C until it the reaction was halted by the user. Negative controls were used to verify the lack of *Taq* performance without the DNA template. Three replicate PCR products of the same sample were pooled in a PCR tube. They were then visualized on agarose gels (2% in TBE [Tris-borate-EDTA] buffer) containing ethidium bromide and purified with a DNA gel extraction kit (AxyPrep DNA; Axygen, China). Prior to sequencing, the DNA concentration of each PCR product was determined using a QuantiFluor TM-ST handheld fluorometer with a UV/blue channel (Promega). The purified amplicons were sequenced using the paired-end method on an Illumina Miseq platform following the PE300 sequencing protocol (Major BioPharm Technology, Shanghai, China).

Sequencing data processing. The sequencing data were subjected to bioinformatic analysis. Prior to analysis, the original data were filtered and optimized to obtain the valid and trimmed sequences through the use of a Trimmomatic trimmer and the FLASH program. Sets of sequences with 97% identity were defined as an operational taxonomic unit (OTU). OTUs were assigned to a taxonomy using the Silva rRNA gene database project in QIIME (14, 15). Rarefaction curves, the Simpson index, the Shannon-Wiener index, the Chao1 estimate, and ACE estimator were also determined using QIIME (16). Community data were generated according to the taxonomic classification data using R tools.

Statistical analysis. The survival of the mice was analyzed by Kaplan-Meier survival analysis. The statistical significance of the results by comparison with those for the untreated infected controls was calculated using the log-rank test. Signs of severity were analyzed as box-and-whisker plots. The data in box-and-whisker plots are expressed as the mean ± standard deviation (SD). Analysis of the nonparametric severity data was performed by one-way analysis of variance (ANOVA), with statistical significance being set at a P value of <0.01. SDs represent the values from at least 3 independent measurements, unless otherwise stated. The unpaired Student t test was used to determine the significance of the differences between mean values (17).

RESULTS

Outcomes after vancomycin treatment in mouse model of CDI. Mice that were exposed to antibiotics and then challenged with *C. difficile* developed clinical symptoms of CDI. In the control infected group (group B), mice showed significant weight loss from day 2 and reached their lowest weight on day 3. By day 3, 80% of mice were moribund and dead. The remaining 20% of the mice that survived showed clinical recovery from diarrhea and other signs of active CDI, marked by weight gain that returned to the baseline by day 13 (see Fig. 3). The toxin A and B ODs in the feces of the surviving mice were increased and peaked on day 1 and then slightly declined during the following 2 weeks. As a comparison, none of the mice in the control uninfected group (group A) showed signs of disease, and all mice in group A survived until the end of the experiment. In the group treated with vancomycin (group C), the treatment against the infection was clearly effective. Mice showed no significant weight loss and an improved mean DAI compared with that of the infected controls (1.73 ± 0.34 versus 2.90 ± 0.15; $P < 0.01$) in the first week, and 90% of mice survived until day 6. However, after the discontinuation of vancomycin, the animals developed signs of CDI and weight loss became progressive from day 6. The mean DAI for vancomycin group mice in the second week after infection was worse than that for the surviving untreated infected mice (2.78 ± 0.19 versus 1.85 ± 0.21; $P < 0.01$). Over time the DAI became progressively worse, followed by weight loss and increased toxin A and B OD levels. Deaths occurred at day 9 in the vancomycin-treated group (Fig. 2). The remaining surviving mice eventually recovered from diarrhea, weight loss, and other signs of CDI. At the end of the experiment, the vancomycin treatment (50 mg/kg/day via gavage for 5 days) led to a 70% overall mortality rate, whereas the overall mortality rate was 80% in the control infected group (Fig. 2).

The addition of berberine treatment prevented the relapse of CDI in vancomycin-treated mice. On day 6, 18 surviving mice treated with vancomycin for 5 days were equally divided into two groups (group C and group D). There was no significant difference in the mean relative weight and the DAI or toxin A and B levels between the two groups. Group D mice were gavaged with berberine (100 mg/kg/day) for 5 consecutive days. As a control, the group C mice were administered the vehicle for 5 days without any therapeutic. The therapeutic efficacy of the berberine added to the treatment regimen was examined. In group D mice, a brief weight loss emerged at day 8 and persisted until day 9 (Fig. 3). At day 10, their weight was restored, and restoration of their weight was accompanied by a decrease in toxin A and B levels. Consistent with the body weight loss, the DAI scores were decreased in group D mice at day 8, and on day 14, the DAI scores were obviously

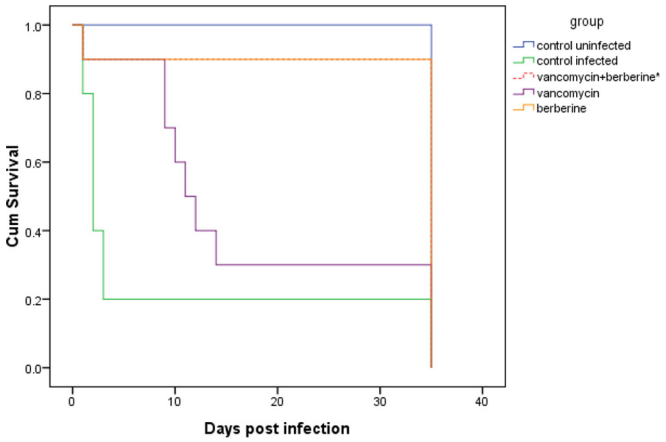

FIG 2 Kaplan-Meier survival plots of mice (exposed to antibiotic pretreatment for 3 days) challenged with *C. difficile* VPI 10463 and subsequently treated on different schedules. *, the line for the combination of vancomycin and berberine coincides with the line for berberine alone; Cum, cumulative.

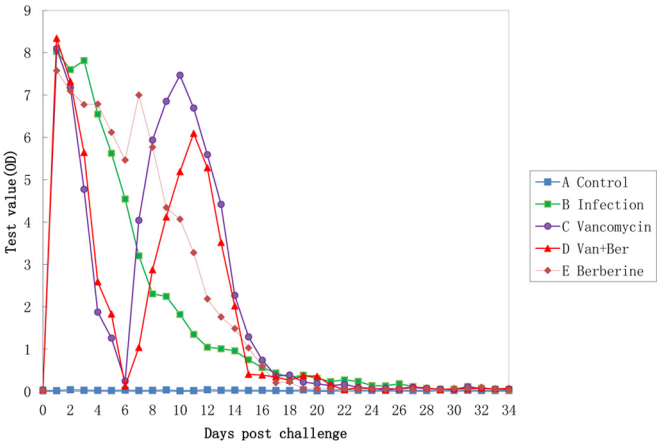

FIG 4 Toxin A and B levels (ODs) in the feces of C57BL/6 mice treated with different regimens. A, control uninfected group; B, control infected group; C, vancomycin-treated group; D, vancomycin-berberine-treated group; E, berberine-treated group.

lower in group D mice than group C mice (group D, 1.37 ± 0.32; group C, 2.78 ± 0.19; $P < 0.05$). In comparison, a relapse emerged in group C mice 2 days after vancomycin treatment was discontinued, and significant weight loss was observed and persisted until day 11, by which time 70% of the mice had died.

Berberine treatment alone for CDI without vancomycin treatment. Group E mice were infected with *C. difficile* and treated with berberine (100 mg/kg/day) alone for 5 days. Significant weight loss emerged on the second day and reached a nadir on the third day. After the last dose of berberine was given, weight loss did not appear again and CDI did not relapse, which is different from the results for the group treated with vancomycin alone. The weight of the surviving mice kept increasing and reached normality on day 15. Compared with the results for the control infected group, berberine therapy significantly reduced the mortality rate in group E mice (10%), consistent with the results for the group treated with vancomycin and berberine.

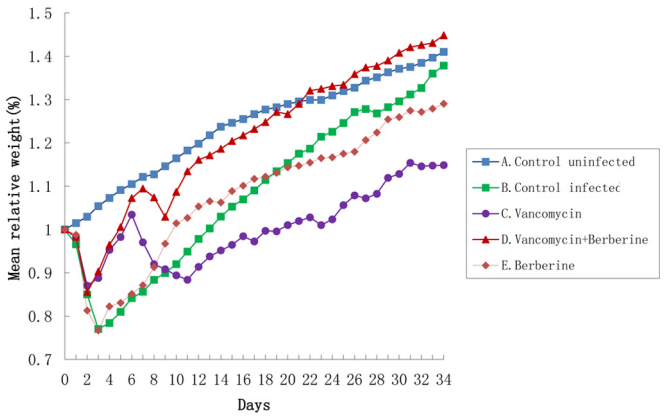

FIG 3 The mean relative weight of all surviving mice was measured during the survival period. The body weights of the mice were measured every day and are presented as a percentage of the original mean body weight on day 0. Mice in the control infected group showed significant weight loss at day 2 and reached their lowest weight on day 3, and then the weight of the surviving mice (20%) kept increasing and reached normality by day 15. The group treated with vancomycin showed an evident weight regain after *C. difficile* inoculation. After the last dose of vancomycin was given, weight loss became evident after day 6 and reached a nadir on day 11. The mice in the group treated with vancomycin and berberine decreased slightly in weight at day 7, and their weights recovered to normal on day 10.

ODs of toxins A and B in feces of C57BL/6 mice in different groups. Fecal samples from mice were examined for *C. difficile* toxins A and B (Fig. 4). Mice that received the antibiotic cocktail and clindamycin prior to *C. difficile* challenge were toxin A and B positive on the day after challenge. After 5 days of vancomycin treatment, the toxin A and B OD value dropped below the detection limit on day 6. In the control uninfected group, the toxin A and B OD value was maintained at a high level for 15 days. When vancomycin was discontinued, toxins A and B were once again detected in the feces. Toxins A and B were also detected in the feces of mice treated with vancomycin combined with berberine, but the level was significantly lower than that in mice treated with vancomycin.

Histopathological changes during various treatment periods. We further investigated the underlying histopathological changes in colonic sites of mice receiving the different treatments during each period of infection and treatment. The sum of the histopathology scores was calculated. Histologic examination of colonic tissues obtained from antibiotic-treated mice exposed to *C. difficile* showed the presence of proliferative ulcerative enteritis with superficial epithelial necrosis and the spread of inflammatory exudates and necrotic cellular material into the intestinal lumen (Fig. 5B). Extensive submucosal edema without submucosal inflammation was prominent, which is also described in human *C. difficile*-associated colitis (18). Moreover, patchy epithelial necrosis, mucosal proliferation, and the presence of inflammatory cells, which are predominantly composed of neutrophils, were found in the tissue. The small intestine was largely spared from cytopathic changes. An initial striking abnormality was the presence of marked submucosal edema and swelling. Colons from mice with CDIs treated with the vehicle contained severe ulceration and inflammatory cell infiltration over the proximal and distal regions (Fig. 5C). In contrast, mice with CDIs treated with berberine showed less inflammation and ulceration (Fig. 5D). To further investigate the underlying changes that infection and antibiotic treatment cause at the site of infection, we collected the histopa-

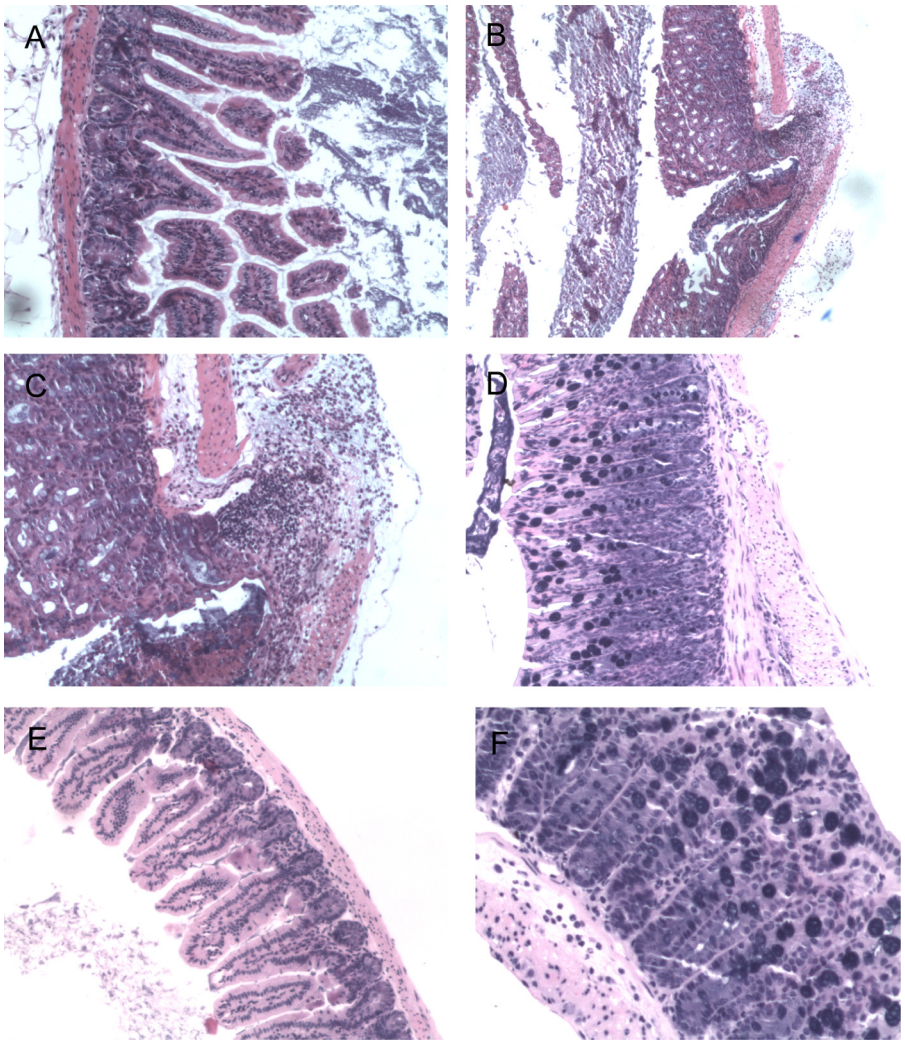

FIG 5 Histology of mouse intestinal tissues collected when mice were moribund or at the times of sacrifice (at the end of each experimental period). Representative H&E-stained colon tissues of mice are shown. (A) Normal colonic tissues of an uninfected control mouse showing a normal configuration of the gland; (B) infected colon at day 3 showing proliferative ulcerative enteritis with superficial epithelial necrosis and the spread of inflammatory exudates and necrotic cellular material into the intestinal lumen; (C) colon of a moribund mouse within 2 days postinfection showing inflammatory cell invasion of the whole layer of the intestinal wall reaching to the serosa and rupture of part of the intestinal canal muscule; (D) colon of a mouse on day 8 after treatment with vancomycin (relapse) showing a scattered distribution of neutrophils in the mucosa lamina propria; (E) colon of a mouse at day 11 treated by addition of berberine after vancomycin treatment showing the submucosa and muscularis mucosa layer and no significant inflammation; (F) colon of a mouse at day 6 treated with berberine treatment alone showing no significant inflammation. Magnifications, ×100.

thology scores for cecum tissues from 3 mice in each group at weeks 1, 2, 3, and 4 postinfection (Fig. 6). As expected, in week 1, untreated infected mice displayed the worst histopathology (8.67 ± 0.58), followed by berberine-treated mice (7.00 ± 1.0), and only minimal changes in the intestinal tissue were seen in vancomycin-treated infected mice (6.33 ± 0.58). By week 2, after the discontinuation of vancomycin, vancomycin-treated mice showed significantly worse histopathology scores than infected control mice (8.00 ± 0.58 versus 7.67 ± 1.53; $P < 0.05$). In the same week, the histopathology scores were markedly reduced in the group receiving berberine after vancomycin treatment (3.67 ± 1.15) and were significantly better in that group than those in mice treated with vancomycin (8.33 ± 0.58). In week 2, the scores in the berberine monotherapy group were also improved (6.00 ± 1.15) compared to the elevated score (6.33 ± 0.58) in week 1. Clearly, the efficacy of berberine monotherapy was limited in comparison with that of the combination of vancomycin and berberine (histopathology scores, 3.67 ± 1.15 versus 6.00 ± 1.15; $P < 0.01$). By week 3, mice receiving the combined treatment showed significantly improved histopathology scores compared with the other groups. Thus, we concluded that berberine therapy could provide clear protection of the colonic mucosa in a mouse model of CDI (12).

Effect of berberine on restoring intestinal microbiota. After removing the reads containing incorrect primer or bar code sequences and sequences with more than one ambiguous base, a total of 320,562 valid reads were obtained and generated through Illumina Miseq PE300 sequencing analysis of 20 samples. Each sample was covered by an average of 16,756 reads. The infected group had the lowest diversity, while samples from normal groups

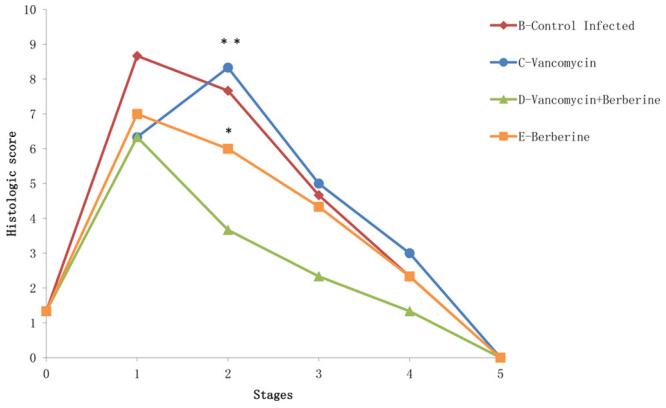

FIG 6 Histopathology scores of cecal tissues of mice in group B (infected without any treatment), group C (infected and treated with vancomycin), group D (infected and treated with a combination of berberine and vancomycin), and group E (infected and treated with berberine). *, $P < 0.01$ for group D compared with group C and group E, determined by two-way ANOVA with Bonferroni's correction; **, $P < 0.05$ for group D compared with group C and group E, determined by two-way ANOVA with Bonferroni's correction.

had the highest PD (phylogenetic diversity) and SI (Shannon diversity index) values. In addition, the PD and SI values for samples from mice in the vancomycin group showed much higher intermouse variations than those for samples from mice in the other groups.

In the GI tract of the normal C57BL/6 mice (control uninfected mice that never received antibiotics), the gut microbial community was dominated by members of the phylum *Firmicutes* (55.55%), with lower proportions of *Bacteroidetes* (35.13%) being found; the remainder was distributed among the *Proteobacteria* (8.81%) (Fig. 7A). The three major phyla detected within the GI tract of the mice were similar to those found previously (16). However, after antibiotic cocktail and *C. difficile* administration, the respective proportions of the three major phyla were altered markedly, with a notable reduction in the proportion of *Bacteroidetes* (0.19%) and an increase in the proportion of *Proteobacteria* (51.29%). At the class level, the *Deltaproteobacteria* and *Epsilonproteobacteria*, which dominated in the control uninfected group (Fig. 7A), were replaced by *Gammaproteobacteria*, which dominated in the infected group (Fig. 7B). Vancomycin could inhibit the growth of *Firmicutes*, but it did not restore the diversity of the microbiota. Vancomycin treatment resulted in a decrease in the proportion of sequences assigned to the phylum *Firmicutes* (9.72%), and this was accompanied by a corresponding increase in the proportion of sequences assigned to members of the *Bacteroidetes* (62.00%) (Fig. 7A). At the class level, the *Clostridia* and *Epsilonproteobacteria* were taken over by bacilli and *Gammaproteobacteria* (Fig. 7C). Although the relative abundance of *Proteobacteria* decreased (26.09%), *Gammaproteobacteria* were still the predominant bacteria noted (Fig. 7B). After 5 days of berberine treatment, regardless of the group (the control infected group or the vancomycin-treated group), the intestinal microbiota of mice experienced significant changes. Berberine inhibited the overgrowth of *Proteobacteria*, especially the *Gammaproteobacteria*, which were replaced by *Deltaproteobacteria* (Fig. 7D and E).

At the order level (Fig. 8), the relative abundance of members of the *Clostridiales* (belonging to the *Clostridia*) and *Enterobacteriales* (belonging to the *Gammaproteobacteria*) was obviously higher in the infected group than in the control group ($P < 0.01$). In contrast, the relative abundance of *Bacteroidales* (belonging to the *Bacteroidetes*) was obviously lower in the infected group than in the control group ($P < 0.01$). Berberine inhibited the growth of *Enterobacteriales* (belonging to the *Deltaproteobacteria*), while there was no effect on *Bacteroidales* or *Clostridiales*. *Campylobacterales* (belonging to the *Epsilonproteobacteria*) and *Clostridiales* (belonging to the *Clostridia*) dominated the normal gut microbiota but were taken over by the *Enterobacteriales* (belonging to the *Gammaproteobacteria*) and *Lactobacillales* (belonging to the bacilli) after vancomycin treatment.

At the family level (Fig. 8), the *Lachnospiraceae* (belonging to the *Clostridiales*) and S24-7 (an unclassified family belonging to the *Bacteroidales*) were dominant in the control group, while in the infected group, *Enterobacteriaceae* (belonging to the *Enterobacteriales*) and *Clostridiaceae* (belonging to the *Clostridiales*) were dominant in the GI tract. After vancomycin treatment, the *Enterobacteriaceae*, *Lactobacillaceae* (belonging to the *Lactobacillales*), and *Porphyromonadaceae* (belonging to the *Bacteroidales*) were dominant in the GI tract. Berberine inhibited the growth of *Proteobacteria* regardless of whether the treatment consisted of berberine alone or berberine in combination with vancomycin, and the proportion of *Deltaproteobacteria* (belonging to the *Proteobacteria*) was significantly higher in both the group treated with berberine alone and the group treated with vancomycin combined with berberine than in the infected group or the group treated with vancomycin alone. At the family level, berberine promoted the growth of the *Bacteroidaceae*, S24-7, and *Lachnospiraceae* (belonging to the *Clostridiales*) but inhibited the growth of the *Enterobacteriaceae*. Thus, berberine could compensate for the gut bacterial imbalance created by vancomycin therapy by restoring the microbiota balance in the GI tract.

DISCUSSION

Berberine is an isoquinoline alkaloid present in numerous plants of the genera *Berberis* and *Coptis*, and it has been used in traditional Eastern medicine for more than 2 millennia. Previous studies have demonstrated the reliable efficacy of berberine in treating bacterial or secretory diarrhea and gastroenteritis. It possesses a wide range of pharmacological and biological activities, including anti-inflammatory and antimicrobial properties (19). The therapeutic benefits of berberine for gastroenteritis and diarrhea have been clearly observed in the clinic, and the mechanisms underlying the efficacy of berberine treatment of colitis have been studied in recent years (6). In this study, we demonstrated that berberine can provide a remedy to an imbalanced gut microbiota, an undesirable consequence created by vancomycin treatment. The high-throughput Illumina Miseq PE300 sequencing approach was used to determine the impacts of berberine therapy on the compositions and diversity of the C57BL/6 mouse intestinal microbiota after vancomycin treatment and to compare the changes in the intestinal microbiota of mice with CDI treated with vancomycin alone and vancomycin in combination with berberine. We provide experimental evidence of the therapeutic efficacy and underlying mechanisms of berberine treatment of CDI in a mouse model of CDI.

Berberine has been shown to have broad significant antimicrobial activity against bacteria, fungi, parasites, worms, and viruses. In particular, berberine has been demonstrated to have highly significant inhibitory activity against *Staphylococcus aureus*, *Strep-*

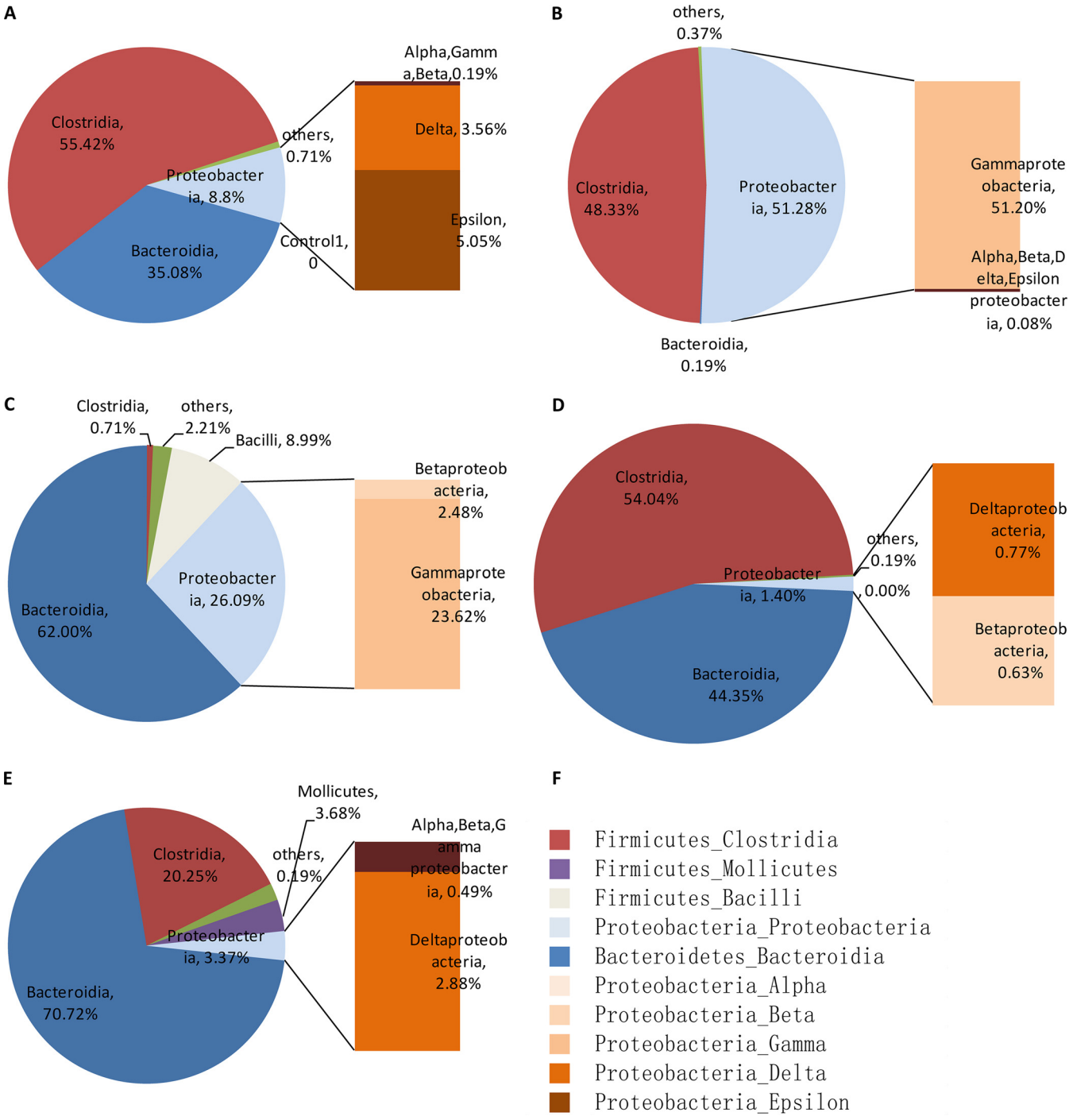

FIG 7 Shifts in microbial community structure and composition associated with *C. difficile* infection and antibiotic administration at the class level.

tococcus, *Salmonella*, *Klebsiella pneumoniae*, *Clostridium*, *Pseudomonas*, *Proteus*, *Shigella*, and *Vibrio* (16). Moreover, berberine has been shown to inhibit the overgrowth of organisms such as staphylococci and coliforms, while it has no significant effect on indigenous lactobacilli and bifidobacteria (9). The significant antimicrobial function of berberine is mediated through its ability to inhibit the assembly of FtsZ (filamenting temperature-sensitive mutant Z) and halt bacterial cell division (9). Berberine is poorly absorbed, acts topically in the gastrointestinal tract, and could modulate the intestinal microbiota without exerting systemic anti-infective activity (16). Berberine has also been shown to inhibit enterobacterial growth in trinitrobenzene sulfonic acid-induced colitis (16).

Normally, the indigenous intestinal microbiota forms a protective barrier against *C. difficile* colonization in the GI tract, but this protective function can be weakened when the host is under-

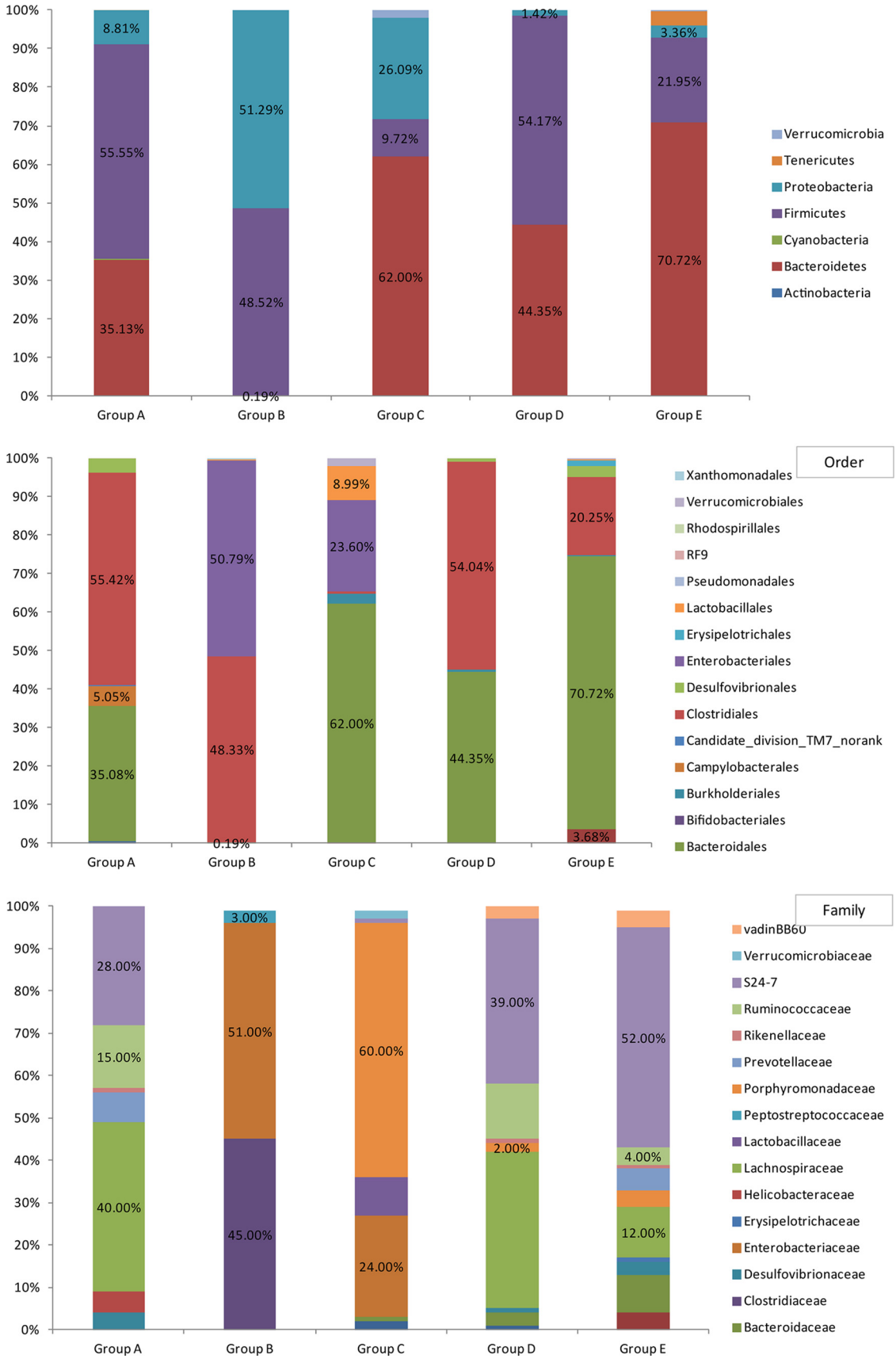

FIG 8 Shifts in microbial community structure and composition associated with *C. difficile* infection and antibiotic administration at the phylum, order, and family classification levels.

going antibiotic therapy (20, 21). Once the host becomes susceptible to pathogens like *C. difficile*, there is a high risk for CDI. Traditional treatments have relied on the use of vancomycin as a bactericidal agent for *C. difficile* (16). However, as a broad-spectrum antibiotic, vancomycin treatment broadly kills bacterial populations and significantly weakens the existent resistance to *C. difficile* colonization by upsetting the normal intestinal microbiota and creating the opportunity for CDI relapse. The main deficiency of vancomycin treatment is its inability to restore the colonization resistance of the normal intestinal microbiota and its inability to resist *C. difficile* spores, leading to CDI relapse. Since vancomycin is poorly absorbed, it is possible that the presence and accumulation of vancomycin may result in ecological disturbances in the intestinal microbiota (22). Furthermore, antibiotic treatment could further aggravate an already imbalanced flora, facilitating the overgrowth of any residual *C. difficile* bacteria or of a newly acquired strain once antibiotics are discontinued. A CDI relapse appears to occur in the setting where the indigenous microbiota is sufficiently disturbed so that colonization resistance cannot be restored even after cessation of the inciting antibiotics and completion of a specific treatment directed against *C. difficile* (16). Our results reveal that vancomycin treatment of mice induced the proliferation of *Enterobacteriaceae* at the expense of other Gram-negative bacteria and of *Firmicutes*. In our studies, vancomycin improved the clinical scores and histopathology in the initial CDI but was associated with a poor prognosis posttreatment in *C. difficile*-infected mice. Our results demonstrate that the most obvious differences were the dominance of *Proteobacteria*, specifically, members of the class *Gammaproteobacteria*, compared to the gut microbial communities found in the control uninfected group versus the control infected group or vancomycin-treated group.

Our study shows that the administration of berberine markedly reduced CDI recurrences in mice after the initial infection, as was verified by tests for toxins A and B and the mitigation of histological damage. The immediate addition of berberine after vancomycin treatment provides the needed remedy to the imbalanced bacterial community in the gut. Our results suggest that berberine inhibits the overgrowth of *Proteobacteria* and restores the imbalanced bacterial community caused by vancomycin, leading to blocking of the CDI recurrence.

Berberine cannot directly inhibit the growth of *C. difficile*, although it can inhibit some bacteria (4). Our data show that berberine monotherapy is not sufficient for the effective control of CDI. Other studies have shown that berberine significantly reduces smooth muscle contraction and intestinal motility and delays the intestinal transit time in humans (23). Therefore, berberine monotherapy in an early phase of a GI tract infection may cause toxin to be stranded in the intestinal tract. Berberine should be avoided as a first step in treating CDI, as it may precipitate toxic megacolon and slow the clearance of *C. difficile* from the intestine. For these reasons, the use of berberine in combination with antibiotics may be more effective for the treatment of CDI.

Previous studies have shown that *C. difficile* spore germination is associated with bile acids (24). Germination is initiated upon exposure to glycine and certain bile acids, e.g., taurocholate. Chenodeoxycholate, another bile acid, inhibits taurocholate-mediated germination (25). As the choleretic activity of berberine stimulates bile and bilirubin secretion (4), we postulate that berberine increases the level of certain bile acids which inhibit *C. difficile* spore germination. A CDI relapse might be inhibited by berberine treatment, although experimental confirmation is warranted. Additional mechanisms underlying berberine's ability to prevent the CDI recurrence include its anti-inflammatory action. Berberine may inhibit the release of arachidonic acid from cell membrane phospholipids and impact arachidonic acid metabolites. An animal study found that berberine could reduce the intestinal secretion of water and electrolytes induced by cholera toxin (26).

The limitations of this study include the experimental design. There should have been another control group that received the antibiotic cocktail but that was not challenged with *C. difficile*. The intestinal flora of mice with CDI should also have been compared to that of mice after treatment with vancomycin or vancomycin plus berberine for 5 days. In the current data set, it is difficult to differentiate the imbalance in the gut flora due to the antibiotic cocktail given before *C. difficile* infection and that due to the subsequent 5 days of vancomycin therapy (with or without berberine). Another limitation is that the study did not adopt the method of fecal *C. difficile* culture. It would have been useful to determine the level of viable vegetative and spore forms of *C. difficile*.

In conclusion, our data suggest that the addition of berberine to the standard vancomycin therapy can restore the upset community structure of the indigenous intestinal microbiota; effectively facilitate recovery from CDI by ameliorating weight loss, reducing the DAI, and improving the histopathology scores in the mouse model of CDI; and block a CDI recurrence. This combinational approach represents a new paradigm in the management of CDI. We provide experimental evidence that the combination of berberine and vancomycin can effectively block a CDI relapse and yield an outcome much better than that achieved with vancomycin alone when it is used to treat CDI in a mouse model of CDI. Therefore, we hypothesize that a berberine-mediated effect could potentially prevent a CDI relapse following vancomycin treatment.

ACKNOWLEDGMENT

The study was supported by Chinese National Natural Science Foundation grant no. 30972819.

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